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Effect of *Eimeria* Infection on Cecal Microbiome of Broilers Fed Essential Oils

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Abstract: Coccidiosis causes mucosal damage and predisposes birds to enteropathogen infection. In this study pyrosequencing was used to evaluate effects of coccidiosis on the intestinal microflora of broilers given diets without feed additives or supplemented with either a growth promotant antibiotic and an ionophore, or two essential oil blends. DNA samples were collected from the cecal contents of broilers before (19 d) and after (26 d) infection with mixed *Eimeria* spp. (*E. acervulina*, *E. maxima* and *E. tenella*). A 454 FLX pyrosequencer and 16S universal primers were used to obtain quantitative profiles of bacterial taxa present in each sample. The relative percent abundance of the identified taxa was analyzed using hierarchical clustering and principal component analysis. Samples from pre-infected broilers were dominated by bacterial species belonging to genera *Subdoligranulum*, *Coprococcus*, *Alistipes*, *Lactobacillus* and *Faecalibacterium*. Post-infection samples were dominated by species from the genera *Escherichia/Shigella* and *Bacteroides*. *Eimeria* infection did not significantly affect the richness of the microbial communities but rather its composition. The composition of the cecal microbiome correlated with the average feed conversion ratio. The methodology used in this study proved effective in understanding the effects of coccidia infection on intestinal microflora of broilers raised on diets supplemented with growth promoting antibiotics, ionophores and essential oils.

Key words: Coccidiosis, microbial ecology, pyrosequencing, essential oils

INTRODUCTION

The importance of elucidating the dynamics of gastrointestinal microbial ecology in farm animals is well recognized (Apajalahti and Bedford, 1999; Apajalahti *et al*., 2004; Dibner and Richards, 2004; Torok *et al*., 2008). Some factors known to influence the intestinal microflora are age, diet, feed processing, feed additives and presence of intestinal pathogens such as *Eimeria* spp. Coccidia are one of the most ubiquitous pathogens that affect broiler production and infection normally involves more than one species (McDougald, 2003). Among other effects, coccidial infection causes mucosal damage and predisposes broilers to dysbacteriosis or clinical illness such as necrotic enteritis (Williams, 2005).

There is a great interest worldwide to develop feed additives which have the ability to positively modulate microflora, improve broiler performance and control pathogens, especially those with zoonotic implications (Thomke and Elwinger, 1998; Verstegen and Williams, 2002; Yang *et al*., 2009). However, the efficacy of a feed additive is influenced by the dose, the way it is applied and many other related factors. One category of feed additives is Essential Oil (EO) blends. These products are mixtures of phytochemicals, such as carvacrol, thymol, eugenol, pipperine and curcumin which can exert a variety of production enhancements including the potential for selective antimicrobial effects (Lee *et al*., 2004; Kim *et al*., 2008). It is thought that such EO blends may improve gut health through a positive modulation of the microflora during coccidial infection (Giannenas *et al*., 2003; Saini *et al*., 2003a; Oviedo-Rondón *et al*., 2006a; Hume *et al*., 2006). Essential oil blends may also decrease *Clostridium perfringens* colonization and proliferation (Mitsch *et al*., 2004) and, consequently, may reduce necrotic enteritis (Saini *et al*., 2003b).

In order to understand the mechanisms of action of new feed additives it is important to monitor the dynamics of gut microflora, determine the key members of the microbiome and establish their functionality (Zoetendal and Mackie, 2005; Antonopoulos *et al*., 2009). Culture-depended methods at the basis of traditional bacterial identification methodologies can be highly exclusionary and selective. The result, for example, is that upwards of 90% of human digestive bacterial species have not been seen nor identified (Zoetendal *et al*., 2004). Culture-independent DNA molecular-based technologies offer relatively less exclusive and less selective taxa

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identification systems. Culture-independent molecular methods can be used to study the dynamics of the intestinal microbial communities (Zoetendal and Mackie, 2005). These techniques are based on either analysis of relative abundance of GC bases, fingerprinting techniques such as PCR-based Denaturing Gradient Gel Electrophoresis (DGGE) or sequencing of clone libraries, providing snap-shots of the microbial community. Previously these techniques were employed to detect shifts in microbial communities in the cecum, ileum and duodenum of birds grown under different management, nutritional and health conditions (Apajalahti and Bedford, 1999; Apajalahti *et al*., 2001; Van der Wielen *et al*., 2002; Hume *et al*., 2003; Amit-Romach *et al*., 2004; Apajalahti *et al*., 2004; Guo *et al*., 2004; Oviedo-Rondón *et al*., 2006a; Parker *et al*., 2007). Hume *et al*. (2006) showed that a major shift in the microbiome following coccidial infection was observed in the cecum. Identification of specific members of the microbiome previously required DNA clone library sequencing which is time-consuming, expensive and error-prone. The advent of the bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) technique (Dowd *et al*., 2008) allows quantitative assessment of the microbiome composition and taxonomic identification of members of the bacterial community, consequently providing a more comprehensive examination of the dynamics of intestinal flora at a level of resolution not previously available.

The present study uses pyrosequencing technique to examine the effects of infection with mixed *Eimeria* spp. on the cecal microbiome of broilers raised on diets without feed additives, or supplemented with either a growth promotant antibiotic and ionophore, or two specific EO blends.

MATERIALS AND METHODS

All procedures involving birds were approved by the Institutional Animal Care and Use Committee of *Stephen F.* Austin State University. The *in vivo* effects of infection with sporulated oocysts from field isolates of mixed *Eimeria* spp. on broilers fed diets without feed additives, or with either antibiotic and ionophore, or two EO blends has been previously described by Oviedo-Rondón *et al*. (2006). This study aimed to extend the work reported in a previous publication (Hume *et al*., 2006) related to effects of these treatments on intestinal microbiota by using pyrosequencing on the same microbial DNA samples to examine the effects of these treatments on specific composition of cecal microflora.

Bird husbandry and treatments: The experimental diets and rearing of the broilers were described by Oviedo-Rondón *et al*. (2006). Briefly, one hundred and eighty 1-d-old Cobb 500 male chickens were placed in 24 floor pens (six chickens per pen) and randomly assigned to four dietary treatments: (1) Unmedicated Uninfected (UU) was fed the basal diet without any feed additives and infected at 19 d of age with an inoculum containing mixed *Eimeria* spp., becoming the unmedicated infected control (UI); (2) PC (positive control) was fed basal diet with antibiotic bacitracin methylene disalicylate at 50 g/ton (BMD®, Alpharma, Inc., Ft. Lee, NJ) and anticoccidial ionophore monensin at 90 g/ton (Coban 60®, Elanco Animal Health, Greenfield, IN); (3) CP was fed basal diet with the EO blend Crina® Poultry (DSM Nutritional Products, Inc., Parsippany, NJ) and (4) CA was fed basal diet with EO blend Crina® Alternate (DSM Nutritional Products, Inc., Parsippany, NJ). These EO blends were added to the basal diets in powder form at concentration of 100 ppm. The main compounds within the EO blends are thymol, carvacrol, eugenol and curcumin. These products (CP and CA) vary in the proportions of active ingredient and the exact contents are the proprietary information of DSM Nutritional Products.

Mixed *Eimeria* infection and sample collection: Since most coccidial infections under commercial broiler production involve more than one species (Williams, 2002; McDougald, 2003), we chose to infect broilers with a mixture of the most common *Eimeria* spp. All broilers were infected at 19 d of age with a standard oral inoculum of sporulated oocysts from field isolates of *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella* at 200, 100 and 50 x 10^3 viable oocysts/ml, respectively. Pre-infection samples were collected right before the coccidia infection at 19 d of age and post-infection samples were collected seven days after the infection (26 d of age). In both cases, two chickens from each cage (12 per treatment) were sacrificed and the cecal contents collected and combined. Thus, six combined samples of two birds each were again combined (see below) resulting in combined contents from twelve birds per treatment being analyzed. The cecal samples were frozen in liquid nitrogen and kept at -70°C for further analyses.

DNA extraction and pyrosequencing: The DNA from cecal contents was extracted as described by Hume *et al*. (2006) using QIAamp Mini DNA Kit (Qiagen Inc., Valencia, CA). The DNA from six samples (2 chickens per sample) from each treatment was combined by taking 41.7 ng of DNA from each sample. The final concentration of DNA in each sample was adjusted to 100 ng/µl. A 100-ng (1 µl) aliquot was used for a 50-µl PCR reaction. The 16S universal eubacterial primers 530F (5'-GTG CCA GCM GCN GCG G) and 1100R (5'-GGG TTN CGN TCG TTG) were used for amplifying the 600-bp region of 16S rRNA gene using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA). Sample PCR was

performed under the following conditions: 94°C for 3 minutes followed by 32 cycles of 94°C for 30 seconds; 60°C for 40 seconds and 72°C for 1 minute and a final elongation step at 72°C for 5 minutes. A secondary PCR was performed for 454 Amplicon Sequencing under the same conditions by using specially designed fusion primers with different tag sequences: LinkerA-Tags-530F and LinkerB-1100R (Dowd *et al*., 2008). After secondary PCR, all products were purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA).

In preparation for FLX sequencing (Roche, Nutley, NJ, USA), DNA fragment sizes and concentrations were measured by using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner Biosystems, CA, USA). Nine point six (9.6) million double-stranded DNA molecules/µl with an average size of 625 bp were combined with 9.6 million DNA capture beads and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNA was denatured with NaOH and sequencing primers were annealed. A two-region 454 sequencing run was performed on a 70 x 75 GS Pico Titer Plate (PTP) by using a Genome Sequencer FLX System (Roche, Nutley, NJ, USA). All FLX-related procedures were performed following Genome Sequencer FLX System manufacturers instructions (Roche, Nutley, NJ, USA).

Data analysis: Taxonomic assignment of sequences was done according to confidence values calculated by RDP classifier 2.2 (Wang *et al*., 2007). A sequence was assigned to a taxon at the taxonomy level where the confidence value was more than or equal to eighty. Relative percent abundance of taxa per sample was calculated to form a taxa matrix which was investigated with hierarchical clustering and principal component analysis (PCA) using the *vegan* package (Oksanen *et al*., 2009) of R statistical environment (R Development Core Team 2009). The taxa richness was expressed as the total number of taxa found in a sample. The evenness of the bacterial community was calculated as: H/ln(R) where H is the Shannon diversity index and R is the taxa richness. Differences between the total numbers of taxa in different treatments were assessed using ANOVA test ($P<0.05$).

To explore the overall pattern of similarities between bacterial communities similarities between pairs of samples using the Bray-Curtis dissimilarity index were calculated (Bray and Curtis, 1957), then clustering using average linkage method was performed (Oksanen *et al*., 2010). Detrended correspondence analysis identified a gradient of less than 3 standard deviation units, therefore, a linear method (PCA) was considered to be more appropriate (Ter Braak and Prentice, 1988). To determine the effect of feed additive and *Eimeria* infection on species composition and its relationship with broiler live performance parameters, *envfit* function (available in the *vegan* package) was applied. The effects of group body weight gain, group average feed intake and group average feed conversion ratio on taxa composition were tested. Function *ordisurf* (available in the *vegan* package) was used to fit a surface for significant variables onto ordination. The significance of the amount of variation explained by these parameters was tested with 10, 000 permutations. All statistical analyses were performed in R (http://www.R-project.org).

RESULTS AND DISCUSSION

The results of live performance pre- and post- infection, lesion scores and oocyst counts were reported by Oviedo-Rondón *et al*. (2006). Part of that information will be used to discuss the results of pyrosequencing analyses presented here in. Pyrosequencing of microbial DNA from cecal samples returned 120, 050 sequences. There was no marked difference in sequencing yield or quality between the samples. The average number of sequences per sample was approximately 4, 500, with the majority of the sequences having length of more than 250 bp. Two samples that had less than 2, 000 sequences were removed from the analysis. The RDP Classifier assigned 117, 704 sequences to different taxonomic levels ranging from genus to kingdom. Sequences of less than 50 bp length were discarded. At a confidence level$\geq$80 most of the sequences (71.7%) were classified to the level of genus, 13.9% to family, 8.1% to phylum, 3.8% to class, 2% to order and 0.6% to kingdom. In total, there were 188 distinct taxa. The distribution of the bacterial richness (total number of taxa) in pre- and post-infection samples is shown in Fig. 1.

Two-way ANOVA test for the pre- and post-infection samples showed no effect of infection on the average bacterial richness. There was a significant ($P = 0.04$) effect of feed additives, a tendency ($P = 0.09$) to increase bacterial richness in cecal samples from CP treatment and a significant ($P = 0.003$) increase in diversity and richness of microbial communities in cecal samples of broilers from CA treatment compared to PC samples. These results suggest a positive modulation of the microflora by EO, regardless of *Eimeria* infection, what could cause positive impacts on the broiler host. In fact, chickens fed diets with the EO blend CA had similar feed conversion ratio to the UU broilers 7 days post-infection (Oviedo-Rondón *et al*., 2006).

Hierarchical clustering analysis of the microbial taxa abundance data separated the samples into two large clusters: one cluster included mainly samples from the pre-infection group and the other included samples from the post-infection group (Fig. 2). The pre-infection cluster contained both samples from the UU treatment (black

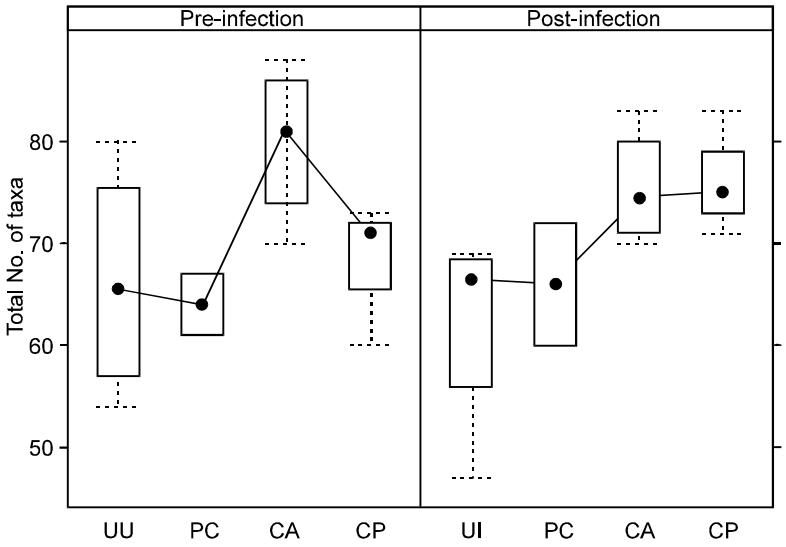

Fig. 1: Box and whisker plots of mean richness of bacterial taxa identified in ceca of broilers pre- and post-infection with mixed *Eimeria* spp. Upper and lower boxplot margins represent the interquartile range and the median is displayed as a black circle. Treatment groups UU = unmedicated uninfected; UI = Unmedicated infected; PC = basal diet supplemented with an antibiotic and ionophore (BMD®+Coban®); CA = Crina® Alternate and CP = Crina® Poultry.

squares), suggesting that uninfected broilers on day 26 had a microbial community composition similar to communities found in pre-infected birds. These results are in agreement with previous findings that demonstrated the effects of *Eimeria* infection on modifying cecal microbial populations (Hume *et al*., 2011, 2012). Additionally Lu *et al*. (2003) reported that changes in cecal bacterial communities between day 14 and 28 were not significant, consistent with our results. The pre-infection group cluster also contained three samples from post-infected groups: Single replicates from UI, CA and PC. The cecal samples in the pre-infection cluster were dominated by bacterial group *Subdoligranulum* (up to 39.2%), *Coprococcus* (up to 31.9%), *Alistipes* (up to 26.2%), *Lactobacillus* (up to 25.6%) and *Faecalibacterium* (up to 23.6%). These numbers are somewhat at odds with the cloning results of Lu *et al*. (2003) from 14-d-old Ross broilers but are in agreement in the relative abundance of *Lactobacillus* (Knarreborg *et al*., 2002; Lu *et al*., 2003). This variance may point to differences in bias between the two molecular techniques (Scupham *et al*., 2007). Meanwhile, Bjerrum *et al*. (2006), using cloning and 16S rDNA gene sequencing of cecal contents from 40-d-old Ross 308 broiler, demonstrated that *Subdoligranulum* and *Faecalibacterium* dominated the chicken cecum. The 16S gene PCR, cloning and sequencing results of Zhu *et al*. (2002) revealed predominance in broiler ceca of *Clostridium leptum* (20.2%), *Sporomusa* sp. (21.2%), *Clostridium coccoides* (27.1%) and enterics (20.8%). The post-infection cluster contained only two samples from the pre-infection group, replicate samples from PC and CA treatments. This cluster was dominated by species from the genera *Escherichia/Shigella* (up to 61.5%) and *Bacteroides* (up to 48.8%). *Bacteroides* were in relative high abundance and *Escherchia coli* were absent in cloned cecal contents from 21- and 28-d-old broilers examined by Lu *et al*. (2003). It was previously shown that these species may play a role in the pathology of cecal coccidiosis (Bradley and Radhakrishnan, 1973). The infection had a strong effect on the dominant taxa and thus the overall composition of microbial communities.

The experimental parameters were fit into PCA ordination in order to determine the effect of feed additives and infection and to observe correlations with broiler live performance. The PCA ordination plot (Fig. 3) displays groups of samples with similar taxon composition closer together and dissimilar samples farther apart. The samples from the pre- and post-infection groups are connected with lines to their geometric center on the ordination space. The first axis explained 54.7% and the second axis 10% of the variance present in the data. The first axis corresponded to mixed *Eimeria* infection (P = 0.0007) separating pre-infection group samples on the right side (white symbols) of the ordination diagram and the post-infection samples (gray symbols) on the left. These results confirm the conclusions of Hume *et al*. (2006) that indicated strong changes in microflora with mixed *Eimeria* spp. infection using the Denaturating Gradient Gel Electrophoresis (DGGE). Also some overlap on the

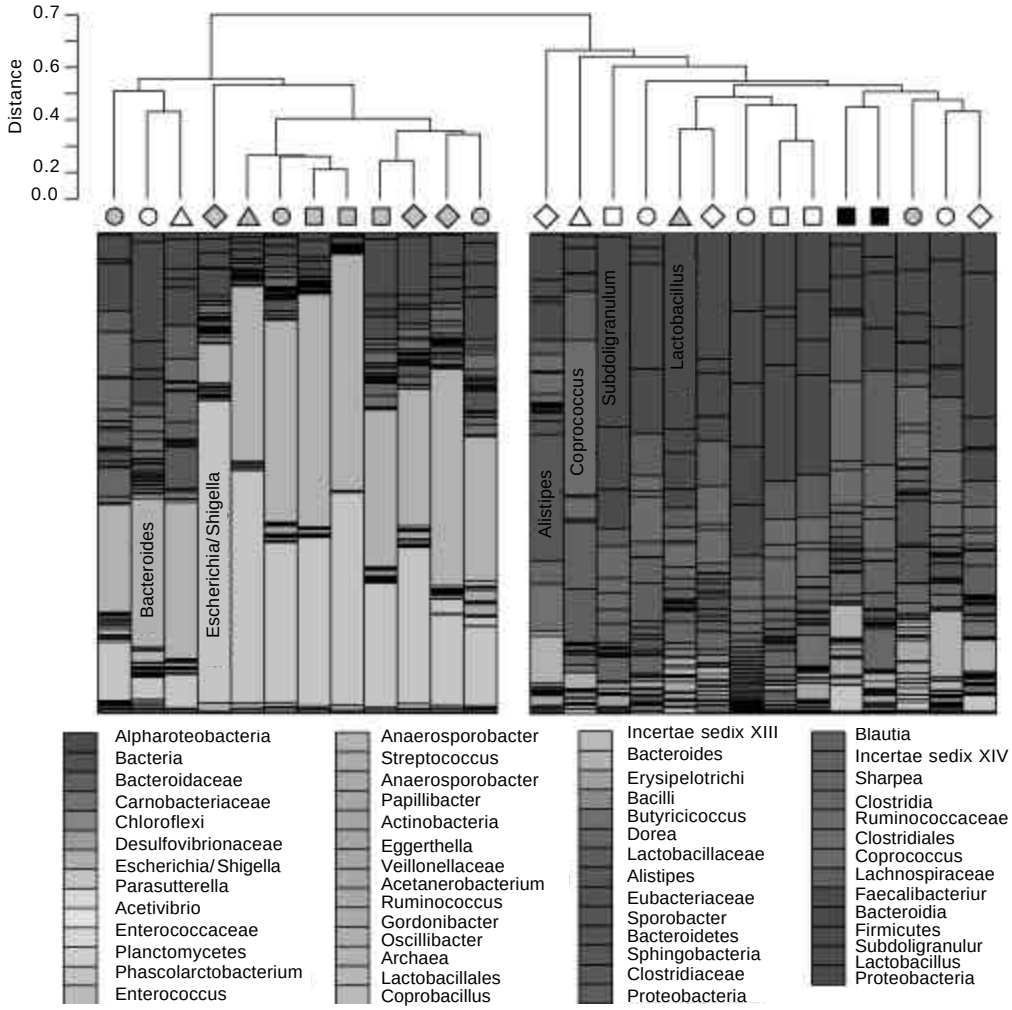

Fig. 2: Hierarchical clustering of the cecal bacterial communities of pre- and post-infection birds. The symbols shown at the bottom of the cluster nodes represent treatments: unmedicated diet = squares; PC, basal diet supplemented with an antibiotic and ionophore (BMD®+Coban®) = triangles; CP, Crina® Poultry = diamonds; CA, Crina® Alternate = circles. White symbols = pre-infection, gray circles = post-infection, black symbols = pre-infection control (UU). The stacked bar chart below the symbols represents the bacterial abundance. Names of bacteria inside the columns represent sequences that could only be identified to that level as opposed to belong to Achaea. The space between the bar charts emphasizes the separation of two main branches of the cluster. The bar to the left indicates the similarity distance between bar charts

ordination can be observed: Single samples from CA and PC post-infection overlap with the pre-infection cluster on the right side of the ordination. In addition, post-infection samples from the unmedicated group were located on the pre-infection group. These results were consistent with the result obtained by hierarchical clustering; the separation of the samples by infection was not complete. Fitting of performance data onto the ordination identified the average feed conversion ratio to significantly correlate with cecal bacterial community composition. Pre-infection samples displayed an average feed conversion ratio of ~1.6 to 1.9, whereas post-infection samples were ~2-2.4. These results suggest that high abundance of *Bacteroides*, *Escherichia* and *Shigella* taxa in the ceca correlate to a higher average feed conversion ratio. Animals with low digestibility and absorption of nutrients may have higher proliferation of those type of bacteria in the ceca, probably due to more undigested nutrients available in the hindgut. Other variables such as feed additives, body weight gain and group average feed intake were not significantly related to the distribution of bacterial

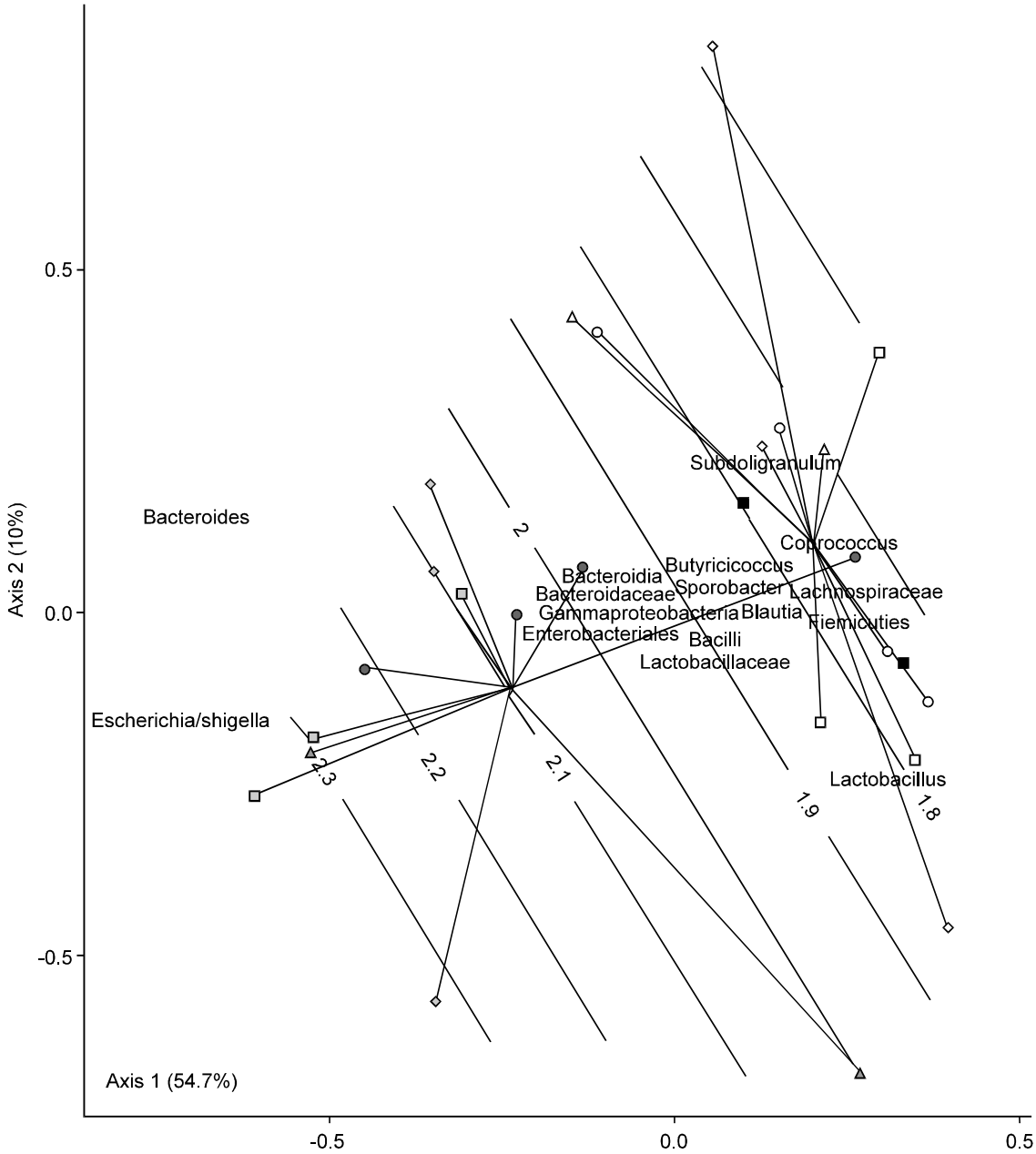

Fig. 3: Principal component analysis showing the relationship between cecal bacterial populations in pre- and post-infection birds from different treatment groups. Treatment groups are represented as: squares = unmedicated diet (white = pre-infection, gray = post-infection, black = post-infection uninfected, UU); triangles = basal diet supplemented with antibiotics (PC) (white = pre-infection, gray-post-infection); diamonds = Crina® Poultry (CP) (white = pre-infection, gray = post-infection); circles = Crina® Alternate (CA) (white = pre-infection, gray = post-infection). Solid lines group samples from pre- and post-infection groups. A contour surface displays a gradual change in the average feed conversion ratio. Only taxa significantly contributing to the ordination graph are shown

community in the ordination space. Moreover, the second PCA axis does not correlate with any explanatory variables and thus was more difficult to interpret.

Previously, our group had verified with using DGGE that these EO blends modulated microbial communities better than PC resulting in less drastic shifts after mixed coccidia infection (Hume *et al*., 2006). However, these results were obtained with a larger number of samples. The positive effects of EO blends can be observed on the ordination plot (Fig. 3). Post infection samples from

CA treatment (gray circles) are located horizontally across the ordination: The location of the sample on the right suggests a bacterial composition similar to one from pre-infection group, whereas the sample on the extreme left suggests a composition of microflora similar to samples from the post-infection unmedicated group high where high abundance of *Bacteroides*, *Escherichia* and *Shigella* taxa was observed. This suggests that feed additives can modulate cecum microflora changes such as the one caused by coccidia and possibly reduce the proliferation of pathogens.

To our knowledge, this is the first quantitative analysis of the distribution and abundance of cecal microbial communities of broilers during the periods before and after an infection with mixed *Eimeria* spp. The hierarchical clustering and PCA provided statistical evidence and a concise assessment of the differences and similarities of bacterial microflora present in the ceca of broilers infected with mixed *Eimeria* spp. and fed corn-soybean diets supplemented with antibiotics or two EO blends: CA and CP. The data analysis suggests that *Eimeria* infection results in low taxon evenness (a community where only few dominant species are found in relatively high abundance). In some post-infected samples *Escherichia/Shigella* and/or *Bacteroides* reached more than 60% relative abundance (Fig. 2). The advent of pyrosequencing enables rapid quantitative characterization of microbial communities suggesting that hypothesis regarding effects of experimental conditions can be evaluated with sufficient statistical rigor. As pyrosequencing becomes more economical, larger number of samples can be examined to test the hypothesis that variation within a group of samples is significantly different from that between the groups. This eliminates justifying statistically unsound pooling together of individual samples as recommended by Zhou *et al*. (2007) "to minimize natural individual variation". The incomplete separation of the samples by medication or infection in our study suggests that effects of *Eimeria* infection is not homogeneous across the flock (Rose *et al*., 1984) and to evaluate the effects of environmental perturbation, sufficient number of replicate samples are required. It is important to mention that pyrosequencing, while a powerful method is inherently limited by sample preparation, amplification, sequencing and data analysis. For example, primer biases as well as the incomplete nature of 16S rDNA databases can lead to unreliable abundance estimates and incorrect or incomplete taxonomic identification. As more bacterial communities are sequenced and studied the effects of these limitations will decrease.

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